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Highly efficient uptake of ultrafine mesoporous silica nanoparticles with excellent biocompatibility by *Liriodendron* hybrid suspension cells

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The characteristics of the interactions co-cultures of ultrafine mesoporous silica nanoparticles (MSNs) and the *Liriodendron* hybrid suspension cells were systematically investigated using laser scanning confocal microscope (LSCM) and scanning electron microscopy (SEM). Using fluorescein isothiocyanate (FITC) labeling, the LSCM observations demonstrated that MSNs (size, 5–15 nm) with attached FITC molecules efficiently penetrated walled plant cells through endocytic pathways, but free FITC could not enter the intact plant cells. The SEM measurements indicated that MSNs readily aggregated on the surface of intact plant cells, and also directly confirmed that MSNs could enter intact plant cells; this was achieved by determining the amount of silicon present. After 24 h of incubation with 1.0 mg mL^{-1} of MSNs, the viability of the plant cells was analyzed using fluorescein diacetate staining; the results showed that these cells retained high viability, and no cell death was observed. Interestingly, after the incubation with MSNs, the *Liriodendron* hybrid suspension cells retained the capability for plant regeneration via somatic embryogenesis. Our results indicate that ultrafine MSNs hold considerable potential as nano-carriers of extracellular molecules, and can be used to investigate *in vitro* gene-delivery in plant cells.

mesoporous silica nanoparticles, endocytosis, cytotoxicity, somatic embryogenesis

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Along with developments in the application of nanotechnology from animal science and medical research to plant science research, the impact of engineered nanomaterials on plant systems has attracted increasing attention, the areas including (i) the delivery of fertilizers, herbicides, pesticides and exogenous genes, (ii) the improvement of the growth of plants, and (iii) nanotoxicity research for plant cells [1–10]. However, compared with mammalian cells, the plant cell wall which is composed of cross-linked polysaccharides (cellulose, hemicelluloses, and pectin) represents an extra barrier surrounding the cell membrane that hinders the passage of nanoparticles into plant cells. To avoid the inhibiting effects of the plant cell wall, free protoplasts (which are prepared via the removal of the cell wall using cellulase treatments) have been used previously to study the internalization of various nanoparticles (e.g., CdSe/ZnS quantum dots (QDs), polystyrene nanospheres, poly(phenylene ethynelene) nanoparticles, and carbon nanomaterials) [11–13]. However, protoplast-based transformation methods hold disadvantages in that the viability of the protoplasts and their ability to divide are strongly reduced by the chemicals that are applied to disorganize the cell wall. For this reason, recent studies have focused on intact plant cells; it was found to that they are able to achieve endocytosis to directly internalize single-walled carbon nanotubes (CNTs), CdSe/ZnS QDs, or poly (amidoamine) dendrimer from the

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extracellular environment [14–18]. Other work showed that multi-walled CNTs with attached cellulose are also able to penetrate the cell wall, and transport intracellularly through cellulose-inducing nanoholes [19].

In the past decade, mesoporous silica nanoparticles (MSNs) have shown significant advantages over traditional nanotransporters as *in vivo* delivery systems for exogenous molecules, due to their tailored mesoporous structure, high surface area, and excellent biocompatibility [20–23]. While the intracellular uptake of MSNs into mammalian cells has been widely studied, the investigation of their uptake by plant cells remains a relatively dormant field. There is only one paper reporting that after decoration of their surface with triethylene glycol, MSNs (size, ~200 nm) could be endocytosed by tobacco mesophyll protoplasts [24]. Here, ultrafine MSNs (size, 5–15 nm) showed an enormous capacity to traverse both plant cell walls and cell membranes. Significantly, after 24 h of incubation with large added amounts of MSNs (1 mg mL^{-1}), the plant cells retained high viability, and successfully developed into somatic embryos and emblings via somatic embryogenesis. In contrast with other nanomaterials such as CNTs or CdSe/ZnS QDs that show high cytotoxicity toward plant cells [15,25,26], these ultrafine MSNs with excellent biocompatibility show great potential in the field of plant biotechnology as nano-carriers for the targeted delivery of small molecules, peptides, and nucleic acids to specific organelles or host genomes at the cellular level.

1 Materials and methods

1.1 Preparation of FITC-labeled MSNs (F-MSNs)

MSNs (spherical, porous, 5–15 nm in size (TEM), Sigma, MO, USA) were dispersed in 3∶1 (v/v) concentrated H_2SO_4/ 30% H_2O_2 for 15 min at 80°C; the supernatant was removed after 10 min of centrifugation at 1×10^4 r min^{-1}, and the particles were then re-suspended in water using sonication. After removing the contaminants via rinsing with water, the MSNs were immersed in an aqueous solution of (3-aminopropyl) triethoxysilane (APTES) (Sigma, MO, USA) (1%, v/v, pH 5.5) for 1 h, and this was followed by thorough washing with water. After this APTES treatment, MSNs were subsequently dispersed in an aqueous solution of fluorescein isothiocyanate (FITC) (Sigma, MO, USA) (1 mg mL^{-1}) for 5 h, and were then washed thoroughly with water. The FITC washed into the supernatant was detected using fluorescence spectrometer (Perkin-Elmer LS 55, USA).

1.2 Plant suspension cell cultures

Under 90 r min^{-1} shaking at 23°C in a dark environment, *Liriodendron* hybrid suspension cells were cultured in 0.43% (w/v) Murashige and Skoog (M&S) liquid medium for 1–2 d. After filtration with a 300-mesh size, a well-dispersed cell suspension was obtained. The population density was determined as ~1.5×10^5 cells mL^{-1}, via a direct count using an inverted microscope.

1.3 Co-culture between the plant suspending cells and MSNs

0.1 mL of FITC-labeled MSNs (0.01, 0.05, 0.1, 0.5 and 1.0 mg mL^{-1}) were added to 1 mL of the *Liriodendron* hybrid cells suspension (1.5×10^5 cells mL^{-1}) under 90 r min^{-1} shaking at 23 or 4°C (achieved using incubation on ice). After 3, 6, 12, or 24 h of incubation, the cells were washed three times using centrifugation at 2000 r min^{-1} for 8 min and were then re-suspended in the liquid culture medium for immediate observation using laser scanning confocal microscopy (LSCM) (Leica TCS SL, Germany) (λ_{ex}=(450±10) nm, and λ_{em}=(520±10) nm). The average intracellular fluorescence intensity (measured from 50 plant cells) was determined using LAS AF Lite software. In control experiments, plant cells were incubated with the same amount of FITC under the same conditions as those mentioned above.

1.4 Somatic embryogenesis of plant cells

After 24 h of internalization of 1.0 mg mL^{-1} of the MSNs at 23°C, 1.0 mL of the *Liriodendron* hybrid cells suspensions was diluted to the appropriate level, plated on M-agar plates (MS medium supplemented with 5 g L^{-1} agar and 30 g L^{-1} sucrose), and then cultured at 25°C under intermittent illumination with an intensity of 500–1000 lx (16 h light/8 h dark) [27].

1.5 Fluorescein diacetate (FDA) staining

10 μL of FDA dye (F0240, TCI, Japan) (100 μg mL^{-1}) was added to 1 mL of the *Liriodendron* hybrid cells suspensions, and was incubated in the dark for 10 min at 23°C; this was followed by washing with culture medium, and then LSCM imaging (λ_{ex}=(488±10) nm, and λ_{ex}=(530±10) nm). The average intracellular fluorescence intensity (measured from 50 plant cells) was analyzed using LAS AF Lite software.

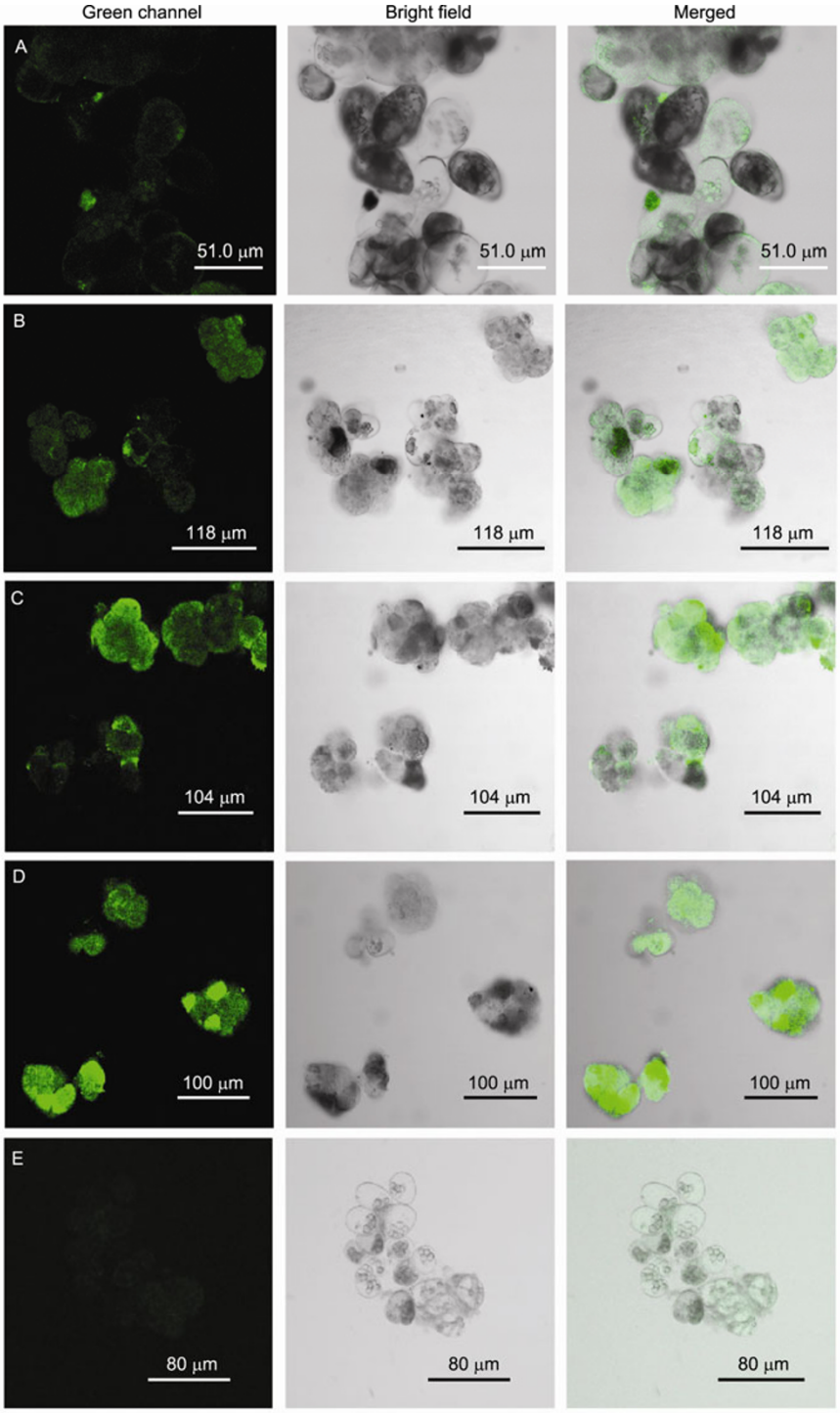
1.6 Scanning electron microscopy (SEM) analysis

The plant cells were dried using a critical point drying method, directly mounted on specimen blocks using two-sided adhesives tape, and then coated with gold for approximately 50 s at 15 mA using an E-100 sputter coater (Hitach, Japan). The SEM images were taken using a QUANTA 200 instrument (FEI Company, Netherlands) with an accelerating voltage of 15 kV. Energy dispersive spectra (EDS) were measured using working voltage of 20 kV, a working distance of 10 mm, and a scanning time of 60 s.

2 Results and discussion

2.1 Uptake of MSNs by Liriodendron hybrids suspension cells

The MSNs were subjected to aminosilanized modification to introduce amino groups to their surfaces (A-MSNs), which were subsequently functionalized using FITC (F-MSNs, λ_{em}=525 nm, photoluminescence (PL) intensity = ~185). The FITC molecules physisorbed on the F-MSNs had to be thoroughly washed with large amounts of water, until no luminescence signal could be detected in the supernatant from FITC (PL intensity<3). To prove that the F-MSNs could enter walled plant cells, *Liriodendron* hybrid suspension cells were used (an important ornamental tree belonging to Liriodendroidae). The cultured *Liriodendron* hybrid suspension cells were incubated in standard growth medium at 23°C with 0.05 mg mL^{-1} of F-MSNs. After being washed with the growth medium, the cells were imaged using LSCM. As shown in Figure 1A–D, after 3 h of



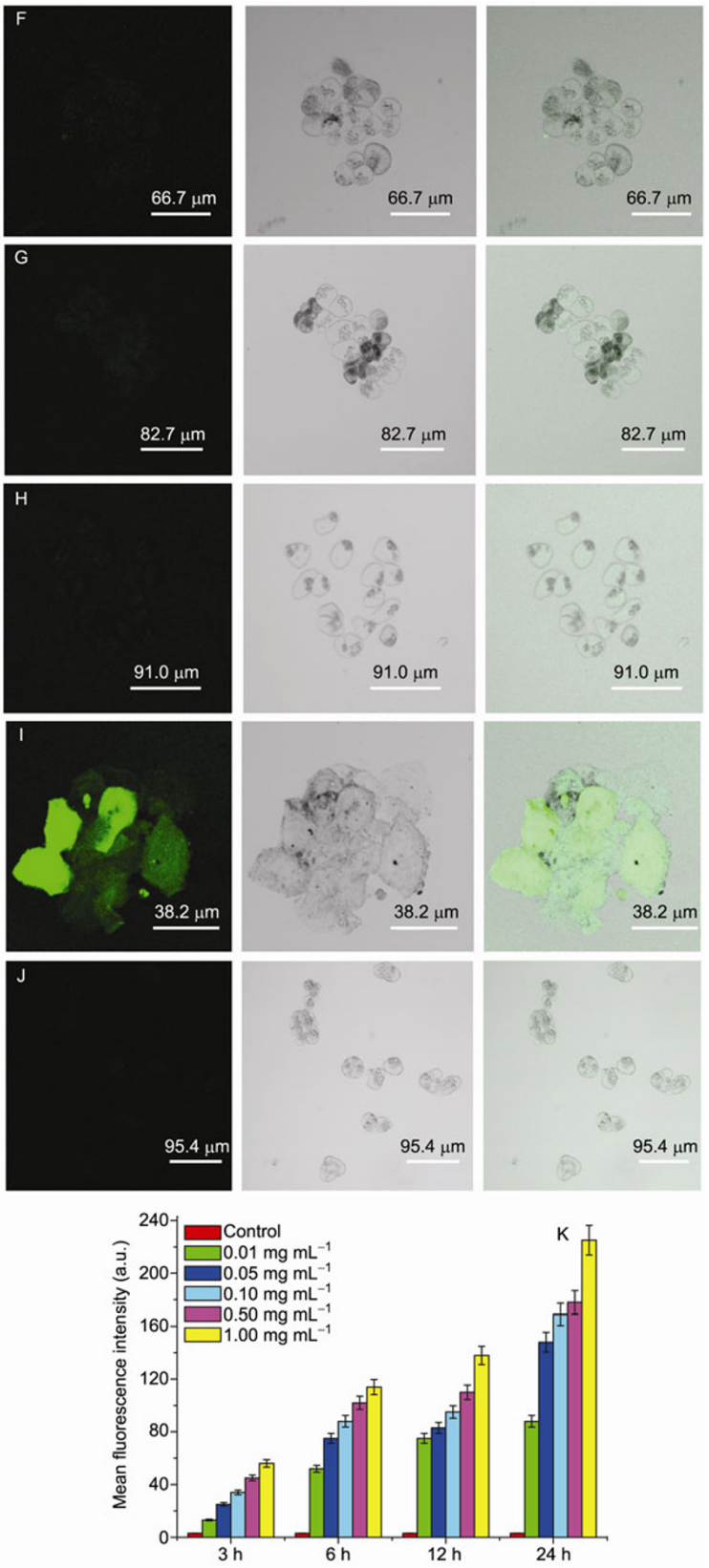


Figure 1 Typical LSCM images of *Liriodendron* hybrid suspension cells incubated with 0.05 mg mL^{-1} of F-MSNs (after (A) 3 h, (B) 6 h, (C) 12 h, and (D) 24 h of incubation), and the control (after (E) 3 h, (F) 6 h, (G) 12 h, and (H) 24 h of incubation with the same amount of added FITC, in the absence of MSNs). I, During control experiments, free FITC could enter the destroyed cells. J, At 4°C, *Liriodendron* hybrid suspension cells incubated with 1.0 mg mL^{-1} F-MSNs after 24 h of incubation. K, Histogram of the intracellular mean fluorescence intensity of F-MSNs with varying concentration, at different incubation times.

co-incubation, the fluorescence signal was most intense near the cell wall. After 24 h of incubation, an intense fluorescence signal could be seen throughout the interior of almost all of the cells. To distinguish the cellular fluorescence signal of the F-MSNs from the FITC signals alone, control experiments were also performed. As a control, plant cells were incubated in growth medium containing the same amount of FITC molecules in the absence of MSNs. From Figure 1E–H, after 24 h of incubation, the cellular fluorescence signal was negligible, compared with that observed with F-MSNs (Figure 1A–D); this showed that free FITC molecules were not easily internalized by intact plant cells. In the control experiments, it was found that free FITC molecules could become concentrated in the destroyed plant cells, because of the loss of cell membrane integrity (Figure 1I). These results confirmed that the strong fluorescence was caused by FITC molecules attached to the MSNs (Figure 1A–D).

We also studied the internalization of MSNs under different conditions. The uptake of F-MSNs was found to be time- and concentration-dependent, as illustrated by Figure 1K. The concentration of F-MSNs in the cell medium was varied, and corresponding increases in the cellular fluorescence were observed. Longer incubation times at 23°C resulted in increases in the fluorescence, while little cellular fluorescence was detected at 4°C, even with high concentrations and long incubation times (Figure 1J). The endocytosis of extracellular molecules has also been proposed for plant cells, but the mechanism nowhere near as well understood as it is for mammalian cells. In our study, the temperature-dependent uptake suggested the involvement of endocytosis for MSNs, since it has been found that endocytosis related to energy in the plant cell is blocked at 4°C [14]. Torney et al. [24] reported that bare MSNs (size, ~200 nm) could not be internalized by plant protoplasts. However, we found that the ultrafine MSNs (5–15 nm) could efficiently penetrate intact plant cells; the particles passed through ~30 nm pores of the plant cell walls, and were then uptaken via cellular endocytosis. We therefore hypothesized that ultrafine MSNs could be also used as nano-carriers to deliver dye molecules (such as FITC molecules) into intact plant cells.

SEM images and EDS spectra were also used to monitor the interactions between the *Liriodendron* hybrid suspension cells and the MSNs (~+18 mV) (or A-MSNs (~−25 mV)). As shown in Figure 2C and E, MSNs and A-MSNs could readily aggregate on the surfaces of the plant cells [28]. Compared with the interior of the plant cells alone (shown in Figure 2B), the concentration of silicon significantly increased from 0.01% to 1.20% (or 6.85%) in the plant cells after the uptake of MSNs or A-MSNs (Figure 2D and F), which directly confirmed the passage of MSNs into the walled plant cells.

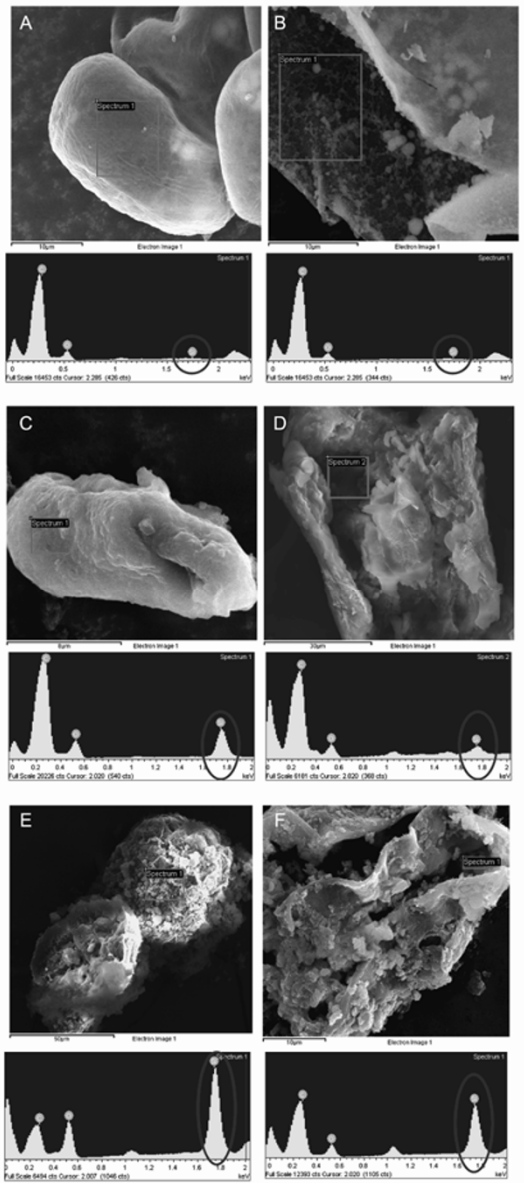


Figure 2 Typical SEM images and EDS energy spectra of *Liriodendron* hybrid suspension cells alone, as a control ((A) the surface of plant cells, (B) the interior of plant cells), *Liriodendron* hybrid suspension cells incubated with 1 mg mL^{-1} of MSNs after 24 h ((C) the surface of plant cells, (D) the interior of plant cells), and *Liriodendron* hybrid suspension cells incubated with 1 mg mL^{-1} of A-MSNs after 24 h ((E) the surface of plant cells, (F) the interior of plant cells).

2.2 Cytotoxicity of MSNs for Liriodendron hybrid suspension cells

FDA hydrolysis assays can be used to determine the viability of plant cells. Living cells will actively convert the non-fluorescent FDA into the green fluorescent compound "fluorescein"; this is a sign of viability, which can be quantified using a fluorescence spectrometer [29]. At 23°C, the cultured *Liriodendron* hybrid suspension cells were incubated for 24 h in the standard growth medium with 1.0 mg mL^{-1} of added A-MSNs or MSNs. After being stained with FDA

dye and then washed with the culture medium, cells were imaged using LSCM at excitation/emission wavelengths of 488/530 nm. As shown in Figure 3A and B, an intense fluorescence signal due to the generation of "fluorescein" could be seen throughout the interior of almost all the cells, which demonstrated that the cells retained high viability

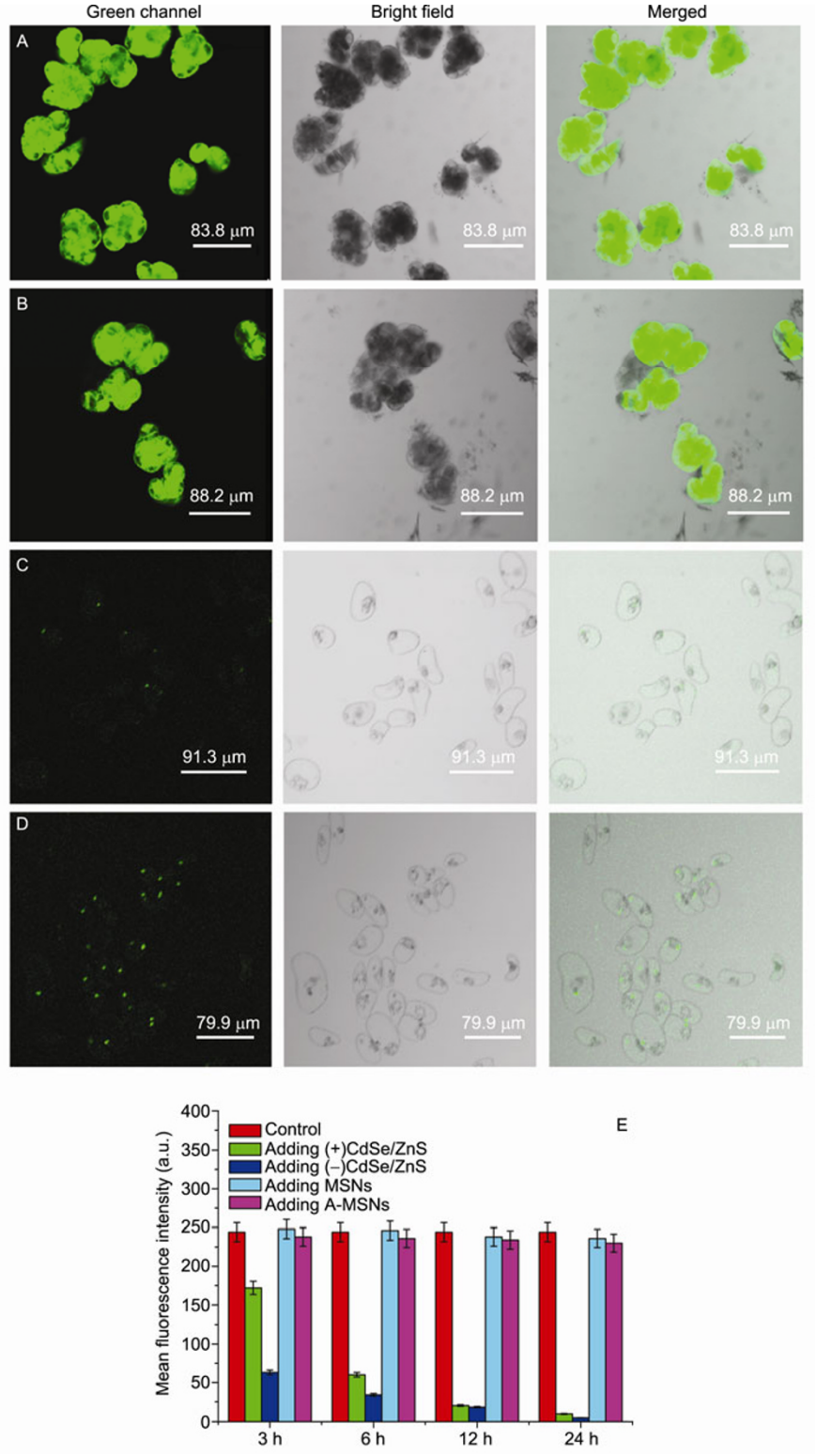


Figure 3 Typical LSCM images of *Liriodendron* hybrid suspension cells (A) with 1.00 mg mL^{-1} of added MSNs after 24 h of incubation, (B) with 1.00 mg mL^{-1} of added A-MSNs after 24 h of incubation, (C) with 1.00 mg mL^{-1} of added (–)CdSe/ZnS after 3 h of incubation, and (D) with 1.00 mg mL^{-1} of added (+)CdSe/ZnS after 6 h of incubation. E, Histogram of the FDA fluorescence intensity of FDA of *Liriodendron* hybrid suspension cells with different added nanoparticles, at different incubation times (untreated *Liriodendron* hybrid suspension cells were used as a control).

after the incubation with A-MSNs or MSNs.

In our previous study on the interactions between CdSe/ZnS QDs and *Liriodendron* hybrid suspension cells [15], for (+)CdSe/ZnS QDs (~20 nm, +15 mV), the apparent death of the plant cells occured after 6 h, and ~94% of the cells lost their viability after 24 h. For (−)CdSe/ZnS QDs (~20 nm, −35 mV), the apparent death of the plant cells occurred after 3 h, and then ~97% of the cells lost their viability after 24 h (Figure 3C and D). In contrast with the investigations of other nanoparticles described in the literature [3,15,25,26], in this study, nearly all of the plant cells retained high viability after the longest incubation time (24 h) with the highest concentration (1 mg mL^{-1}) of MSNs or A-MSNs.

2.3 Somatic embryogenesis of Liriodendron hybrids suspension cells after the internalization of MSNs

Liriodendron hybrid regeneration via somatic embryogenesis was developed in our group; this is a process in which an embryo is derived from a single embryogenic somatic cell or group of embryogenic somatic cells, in contrast with zygotic embryogenesis [27]. The results demonstrating this are shown in Figure 4A–C, and the *Liriodendron* hybrid

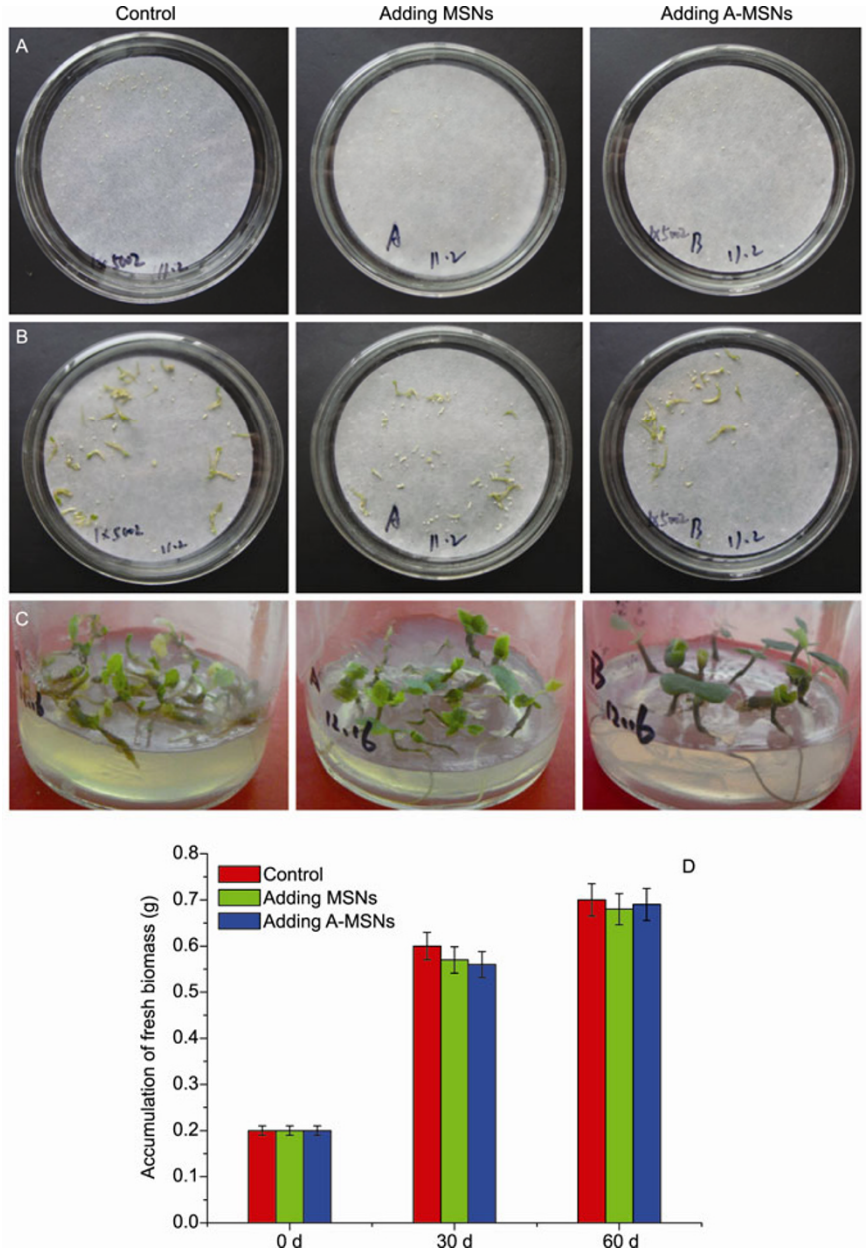


Figure 4 At different times ((A) 20 d, (B) 40 d, (C) 60 d), typical CCD images of somatic embryogenesis of *Liriodendron* hybrid suspension cells after internalizing A-MSNs or MSNs. D, Histogram showing the accumulation of fresh biomass of *Liriodendron* hybrid suspension cells added at different growth times (untreated *Liriodendron* hybrid suspension cells were used as a control).

suspension cells produced after incubation with MSNs or A-MSNs could also be successfully developed into embryos and emblings via somatic embryogenesis. The accumulation of fresh *Liriodendron* hybrid biomass during somatic embryogenesis was also recorded, as shown in Figure 4D; this indicated that the MSNs had no negative effects on the somatic embryogenesis. After incubation with MSNs, the *Liriodendron* hybrid suspension cells retained their capability for plant regeneration via somatic embryogenesis, which showed that these MSNs with excellent biocompatibility held considerable potential for walled plant cells.

3 Conclusion

In conclusion, we found that ultrafine MSNs could be effectively internalized by plant cells via endocytosis. Even after the *Liriodendron* hybrids suspension cells were incubated for 24 h with 1.0 mg mL^{-1} of added MSNs, the plant cells retained their high viability and no cell death was observed. Furthermore, after the *Liriodendron* hybrid suspension cells internalized the MSNs, they retained their capability for plant regeneration via somatic embryogenesis. Compared with other nanomaterials, these ultrafine MSNs with excellent biocompatibility hold considerable potential as nano-carriers for walled plant cells, and could have a major impact in advancing our knowledge of plant cell biology.

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